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Reciprocal Modulation of Toll-like Receptor-4 Signaling Pathways Involving MyD88 and Phosphatidylinositol 3-Kinase/AKT by Saturated and Polyunsaturated Fatty Acids*

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Toll-like receptor-4 (TLR4) can be activated by nonbacterial agonists, including saturated fatty acids. However, downstream signaling pathways activated by nonbacterial agonists are not known. Thus, we determined the downstream signaling pathways derived from saturated fatty acid-induced TLR4 activation. Saturated fatty acid (lauric acid)-induced NFκB activation was inhibited by a dominant-negative mutant of TLR4, MyD88, IRAK-1, TRAF6, or IκBα in macrophages (RAW264.7) and 293T cells transfected with TLR4 and MD2. Lauric acid induced the transient phosphorylation of AKT. LY294002, dominant-negative (DN) phosphatidylinositol 3-kinase (PI3K), or AKT(DN) inhibited NFκB activation, p65 transactivation, and cyclooxygenase-2 (COX-2) expression induced by lauric acid or constitutively active (CA) TLR4. AKT(DN) blocked MyD88-induced NFκB activation, suggesting that AKT is a MyD88-dependent downstream signaling component of TLR4. AKT(CA) was sufficient to induce NFκB activation and COX-2 expression. These results demonstrate that NFκB activation and COX-2 expression induced by lauric acid are at least partly mediated through the TLR4/PI3K/AKT signaling pathway. In contrast, docosahexaenoic acid (DHA) inhibited the phosphorylation of AKT induced by lipopolysaccharide or lauric acid. DHA also suppressed NFκB activation induced by TLR4(CA), but not MyD88(CA) or AKT(CA), suggesting that the molecular targets of DHA are signaling components upstream of MyD88 and AKT. Together, these results suggest that saturated and polyunsaturated fatty acids reciprocally modulate the activation of TLR4 and its downstream signaling pathways involving MyD88/IRAK/TRAF6 and PI3K/AKT and further suggest the possibility that TLR4-mediated target gene expression and cellular responses are also differentially modulated by saturated and unsaturated fatty acids.

Toll-like receptors (TLRs)[1] play a critical role in inducing innate immune responses in mammals by recognizing conserved pathogen-associated molecular patterns of bacteria (1–4). So far, 10 human TLRs have been cloned (5–10). The TLR agonists include lipopolysaccharide (LPS) for TLR4, peptidoglycan for TLR2 and TLR6, double-stranded RNA for TLR3, flagellin for TLR5, and imidazoquinolines and unmethylated CpG motifs in bacterial DNA for TLR7 and TLR9, respectively (3, 11–14). TLR4 can be activated by nonbacterial agonists such as HSP60, fibronectin, Taxol, respiratory syncytical virus coat protein, and saturated fatty acids (15–20).

TLRs are type I transmembrane receptors characterized by the presence of extracellular leucine-rich repeat motifs and a cytoplasmic Toll/interleukin-1 receptor (TIR) homology domain, which is required for the activation of downstream signaling pathways leading to the activation of nuclear factor-κB (NFκB) (21). Myeloid differentiation factor-88 (MyD88) is known as an immediate downstream adaptor molecule that interacts directly with the TIR domain of TLRs (22, 23). MyD88 recruits interleukin-1 receptor-associated kinase (IRAK) and tumor necrosis factor receptor-associated factor-6 (TRAF6), leading to activation of NFκB and mitogen-activated protein kinases (MAPKs) (24, 25). Activation of NFκB leads to the expression of target genes, including cyclooxygenase-2 (COX-2) and cytokines. TIR domain-containing adaptor protein (TIRAP)/MyD88 adaptor-like (Mal) is another adaptor molecule cooperating with MyD88, leading to activation of IRAK-1 and NFκB (26–28). TIR domain-containing adaptor inducing interferon-β (TRIF)/TIR domain-containing adaptor molecule-1 (TICAM-1) has been reported as another adaptor molecule responsible for the MyD88-independent signaling pathway derived from TLR3, leading to the activation of interferon regulatory factor-3 and the expression of interferon-β (29–31). Thus, individual TLR agonist can activate different downstream signaling pathways,

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[1] The abbreviations used are: TLRs, Toll-like receptors; LPS, lipopolysaccharide; TIR, Toll/interleukin-1 receptor; NFκB, nuclear factor-κB; MyD88, myeloid differentiation factor-88; IRAK, interleukin-1 receptor-associated kinase; TRAF6, tumor necrosis factor receptor-associated factor-6; MAPK, mitogen-activated protein kinase; COX-2, cyclooxygenase-2; TIRAP, Toll/interleukin-1 receptor domain-containing adaptor protein; Mal, MyD88 adaptor-like; TRIF, TIR domain-containing adaptor inducing interferon-β; TICAM, TIR domain-containing adaptor molecule; PI3K, phosphatidylinositol 3-kinase; NIK, NFκB-inducing kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; DN, dominant-negative; CA, constitutively active; DHA, docosahexaenoic acid; CBP, cAMP-responsive element-binding protein.

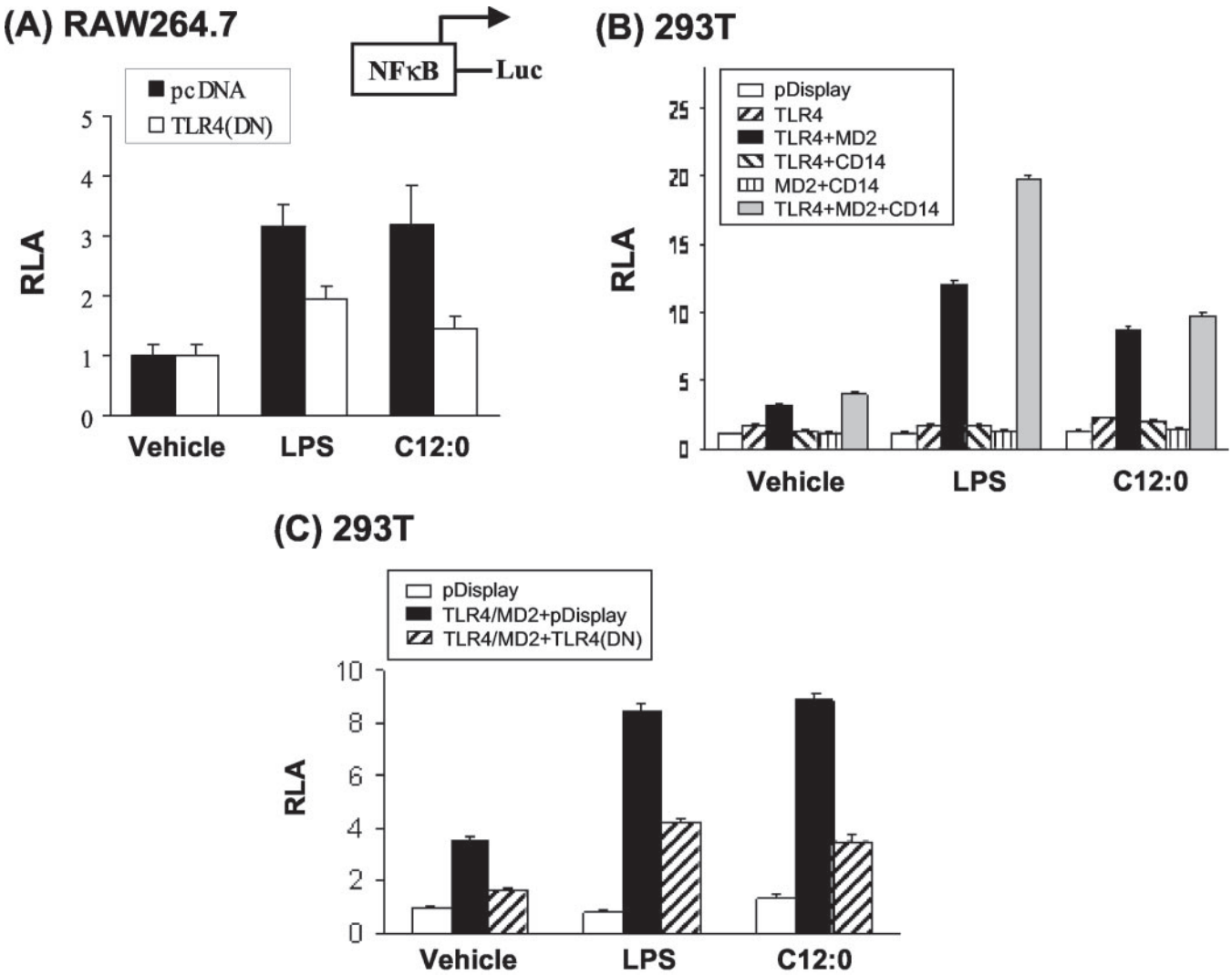

FIG. 1. **Lauric acid (C12:0)-induced NFκB activation is inhibited by a dominant-negative TLR4 mutant in macrophages (RAW264.7) and 293T cells transfected with TLR4 and MD2.** *A*, RAW264.7 cells were transfected with the $(NF\kappa B)_2$-luciferase (*Luc*) reporter plasmid and the expression plasmid of TLR4(DN) or a corresponding vector. Cells were further treated with LPS (200 ng/ml) or lauric acid (75 μM) for 18 h. *B* and *C*, 293T cells were transfected with the $(NF\kappa B)_2$-luciferase reporter plasmid and the expression plasmid of wild-type TLR4, MD2, CD14, or TLR4(DN) as indicated. Cells were further treated with LPS (100 ng/ml) or lauric acid (50 μM) for 18 h. Cell lysates were prepared, and luciferase and β-galactosidase enzyme activities were measured as described under "Experimental Procedures." Relative luciferase activity (*RLA*) was determined by normalization to β-galactosidase activity. Data are representative of more than three independent experiments. Values are means ± S.E. (n = 3).

leading to differential target gene expression and cellular responses.

Phosphatidylinositol 3-kinase (PI3K) and AKT have been implicated in TLR4 and TLR2 signaling pathways. LPS, a TLR4 agonist, activates PI3K, resulting in the phosphorylation of AKT, a downstream target of PI3K (32, 33). It has been demonstrated that TLR2 can associate with Rac1 and PI3K, leading to the activation of AKT (34). AKT, also known as protein kinase B, is a serine/threonine kinase that is activated in response to cytokines and growth factors (35, 36). AKT is activated via phosphorylation by PI3K and further phosphorylates its downstream signaling molecules, including glycogen synthase kinase-3, BAD, and caspase-9 (37–39). AKT has been shown to induce p65 phosphorylation, resulting in enhanced NFκB transactivation (40–44). However, it has not been clearly understood how PI3K/AKT is linked to TLR4 and what the role of PI3K/AKT is in activating downstream signaling pathways of TLR4.

Lipid A, which possesses most of the biological activities of LPS, is acylated with saturated fatty acids. Removal of these acylated saturated fatty acids from lipid A not only results in complete loss of endotoxic activity, but also makes lipid A act as an antagonist to native lipid A (45, 46). Lipid A containing unsaturated fatty acids is also known to be nontoxic or to act as an antagonist against endotoxin (47, 48). These results suggest that the fatty acids acylated in lipid A play a critical role in ligand recognition and receptor activation for TLR4. Results from our previous studies demonstrated that saturated fatty acids induce NFκB activation and the expression of COX-2, but that unsaturated fatty acids inhibit LPS-induced NFκB activation and the expression of COX-2 and other inflammatory markers in a murine monocytic cell line (RAW264.7) by modulating the activation of TLR4 (18).

However, the signaling pathways leading to NFκB activation by saturated fatty acids are not known. Identifying signaling pathways activated by different TLR agonists can help us to understand the mechanisms by which each agonist regulates the expression of target genes and consequent cellular responses. Therefore, in this study, we determined whether TLR4-derived downstream signaling pathways are modulated by saturated and polyunsaturated fatty acids.

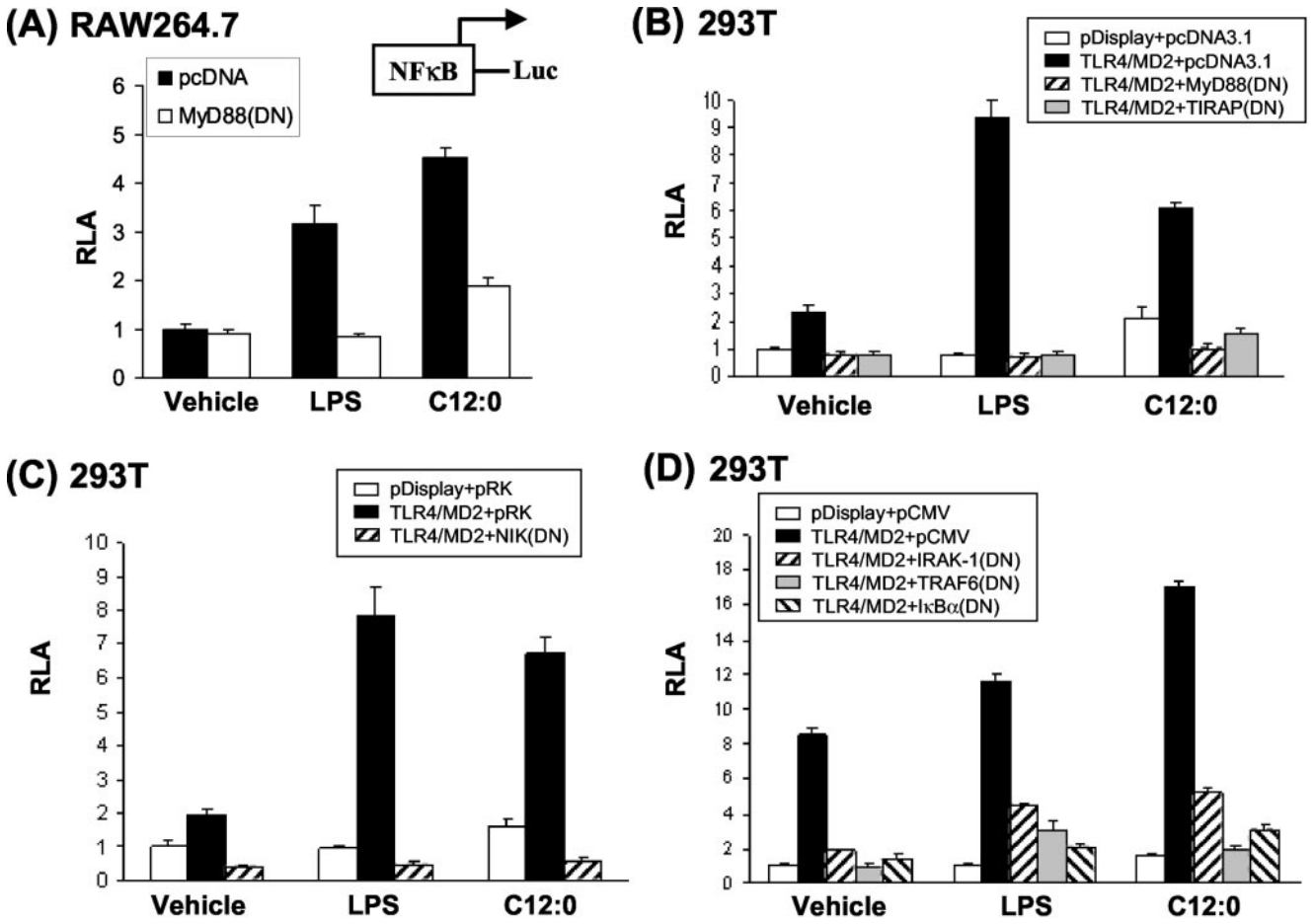

FIG. 2. **Lauric acid (C12:0)-induced NFκB activation is inhibited by a dominant-negative mutant of MyD88, IRAK, TRAF6, NIK, or IκBα.** *A*, RAW264.7 cells were cotransfected with the $(NF\kappa B)_2$-luciferase (*Luc*) reporter plasmid and the expression plasmid of MyD88(DN) or a corresponding vector. Cells were further treated with LPS (200 ng/ml) or lauric acid (75 μM) for 18 h. *B–D*, 293T cells were transfected with the $(NF\kappa B)_2$-luciferase reporter plasmid and the expression plasmid of wild-type TLR4 and MD2. A dominant-negative mutant of each signaling component or a corresponding vector was cotransfected as indicated. Cells were further treated with LPS (100 ng/ml) or lauric acid (50 μM) for 18 h. Cell lysates were prepared, and luciferase activities were determined as described in the legend for Fig. 1. Data are representative of more than three independent experiments. Values are means ± S.E. (n = 3). *RLA*, relative luciferase activity.

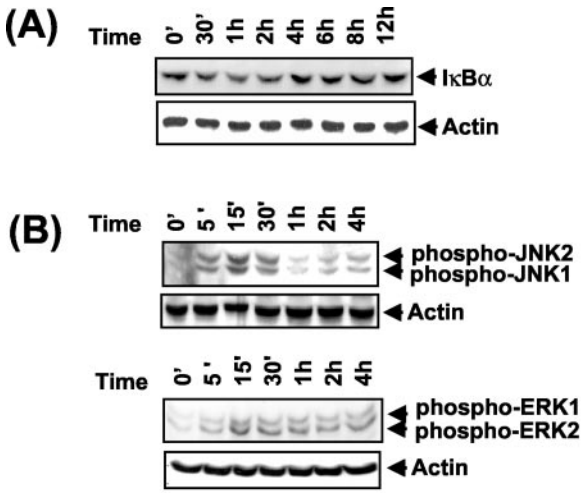

FIG. 3. **Lauric acid (C12:0) induces the degradation of IκBα and the phosphorylation of JNK and ERK in macrophages.** RAW264.7 cells were treated with lauric acid (100 μM) for the indicated times, and cell lysates were analyzed by anti-IκBα (*A*) or phospho-specific anti-JNK and anti-ERK (*B*) immunoblotting. Data are representative of more than two independent experiments.

EXPERIMENTAL PROCEDURES

Reagents—Sodium salts of unsaturated and saturated fatty acids were purchased from Nu-Chek (Elysian, MN) and were dissolved in endotoxin-free water. LPS was purchased from Difco. LY294002 (2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one) was purchased from BIOMOL Research Labs Inc. (Plymouth Meeting, PA). Polyclonal antibodies for COX-2 and glyceraldehyde-3-phosphate dehydrogenase were prepared and characterized as described previously (49, 50). Antibodies for AKT and phospho-Ser^{473} AKT were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-β-actin antibody (ab6276) was obtained from Abcam (Cambridge, UK). All other reagents were purchased from Sigma unless indicated otherwise.

Plasmids—The expression plasmids for a constitutively active form of TLR4 (ΔTLR4) and a dominant-negative mutant (ΔTLR4(P712H)) were prepared as previously described (10). The $(NF\kappa B)_2$-luciferase reporter construct was provided by Frank Mercurio (Signal Pharmaceuticals, San Diego, CA). The luciferase reporter plasmid (pGL2) containing the promoter region of the murine COX-2 gene (−3.2 kb) was a kind gift from David Dewitt (Michigan State University, East Lansing, MI). The HSP70-β-galactosidase reporter plasmid was from Robert Modlin (University of California, Los Angeles, CA). Wild-type TLR4 (pDisplay-TLR4) was obtained from Adeline Hajjar (University of Washington, Seattle, WA). A dominant-negative mutant of p85 (pSG5-p85ΔiSH2) and a dominant-negative mutant of AKT (SRα-AKT(T308A/S473A)) were obtained from Bing-Hua Jiang (West Virginia University).

The expression plasmid of MD2 was obtained from Kensuke Miyake (Saga Medical School, Saga, Japan). CD14 was provided by Richard J. Ulevitch (Scripps Research Institute). A constitutively active form of AKT (myristoylated AKT) and wild-type AKT were provided by Michael Weber (University of Virginia Health Sciences Center). A constitutively active form of MyD88 (MyD88(ΔToll)) and the dominant-negative mutant MyD88(ΔDD) were kindly provided by Jurg Tschopp (University of Lausanne, Lausanne, Switzerland). Constitutively active chimeric CD4-TLR4 was obtained from C. A. Janeway, Jr. (Yale University, New

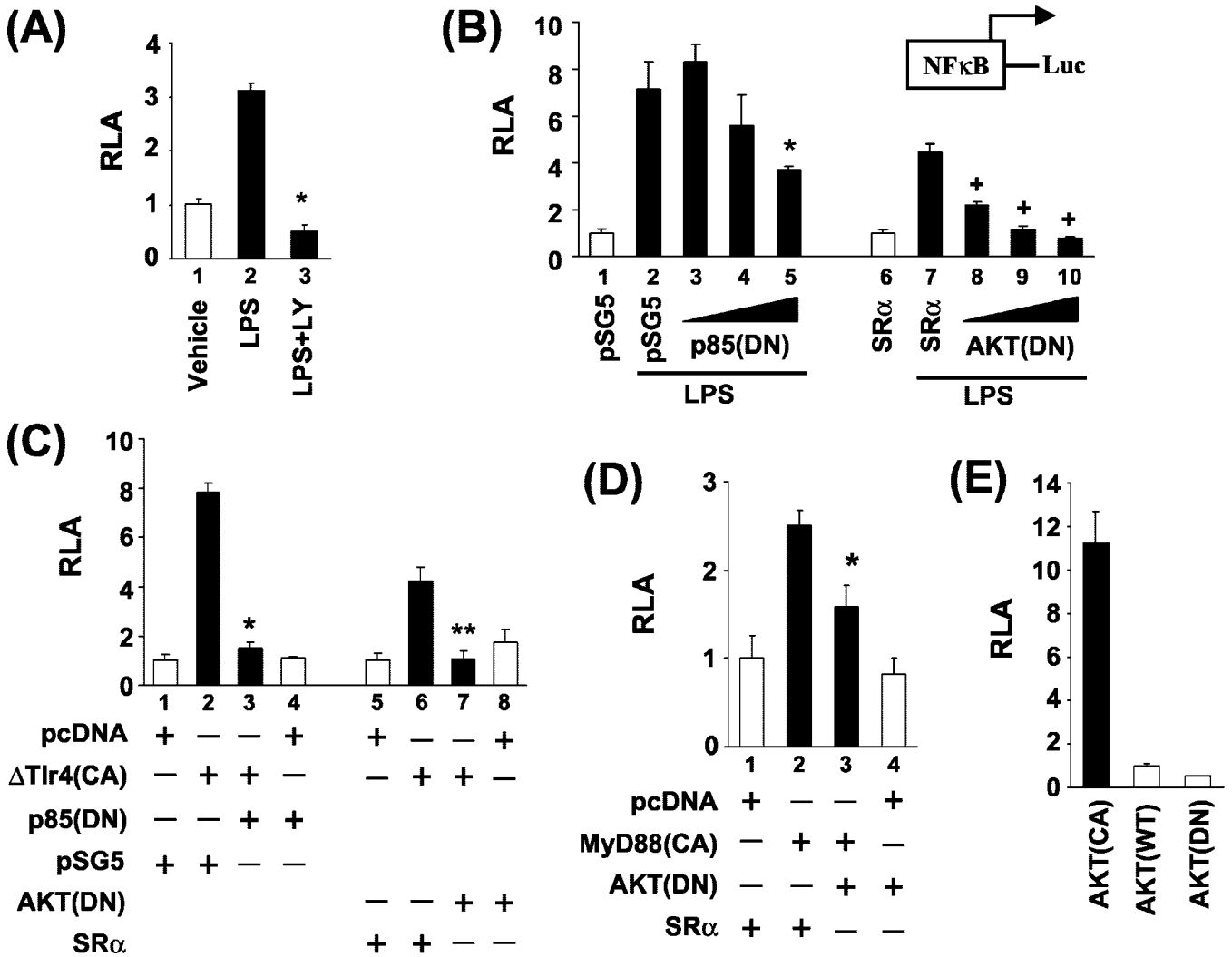

FIG. 4. **LPS- or TLR4(CA)-induced NFκB activation is inhibited by a PI3K inhibitor (LY294002) or a dominant-negative mutant of PI3K or AKT in macrophages.** RAW264.7 cells were transfected with the $(NF\kappa B)_2$-luciferase (*Luc*) reporter plasmid. Cells were pretreated with LY294002 (*LY*; 10 μM) for 30 min (*A*) or were cotransfected with the expression plasmid of a dominant-negative mutant of PI3K (p85(DN)) or AKT(DN) (1, 2, and 3 μg) or a corresponding vector as indicated (*B*) and further stimulated with LPS (100 ng/ml) for 18 h. Cells were cotransfected with the expression plasmid of ΔTLR4(CA) (*C*) or MyD88(CA) (*D*) together with p85(DN) or AKT(DN) as indicated. Cells were cotransfected with AKT(CA), wild-type AKT (*AKT(WT)*), or AKT(DN) (*E*). After 24 h, cell lysates were prepared, and luciferase activities were determined as described in the legend for Fig. 1. Data are representative of more than three independent experiments. Values are means ± S.E. (n = 3). *, **, and +, significant differences from *bars 2*, *6*, and *7*, respectively ($p < 0.05$). *RLA*, relative luciferase activity. SRα, vector for AKT(DN).

Haven, CT). Dominant-negative mutants of TIRAP and TRAF6 (pCMV4-TRAF6-(300–524)) were provided by Ruslan Medzhitov (Yale University School of Medicine). Dominant-negative IRAK-1 (pCMV4-IRAK-1-(1–211)) was a kind gift from Sankar Ghosh (Yale University School of Medicine). A dominant-negative mutant of NFκB-inducing kinase (NIK) was a gift from M. Rothe (Tularik, South San Francisco, CA). A dominant-negative mutant of IκB (pCMV4-IκBα(ΔN)) was provided by Dean Ballard (Vanderbilt University, Nashville, TN). All DNA constructs were prepared in large-scale using the EndoFree plasmid maxi-kit (QIAGEN Inc., Chatsworth, CA) for transfection.

Cell Culture—RAW264.7 cells (a murine monocytic cell line, ATCC TIB-71) and 293T cells (human embryonic kidney cells; provided by Sam Lee, Beth Israel Hospital, Boston, MA) were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) heat-inactivated fetal bovine serum (Intergen Co.), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C in a 5% CO_2 and air environment.

Transient Transfection and Luciferase Assay—These were performed as described in our previous studies (10, 18, 51). Briefly, RAW264.7 or 293T cells were plated in 24-well plates (1.5×10^5 cells/well) and cotransfected with a luciferase plasmid containing either the $(NF\kappa B)_2$-binding site or the murine COX-2 promoter (−3.2 kb) and with the HSP70-β-galactosidase plasmid as an internal control using SuperFect transfection reagent (QIAGEN Inc.) according to the manufacturer's instructions. Various expression plasmids or corresponding empty vector plasmids for signaling components were cotransfected. The total amount of transfected plasmids was equalized by supplementing with the corresponding empty vector to eliminate the experimental error from transfection itself. Luciferase and β-galactosidase enzyme activities were determined using the luciferase assay system and the β-galactosidase enzyme system (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized to β-galactosidase activity.

Immunoblotting—This was performed as previously described (52, 53). Briefly, solubilized proteins were subjected to 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was blocked in 20 mM Tris-HCl, 137 mM NaCl, and 0.05% (v/v) Tween 20 (pH 7.6) containing 5% nonfat dried milk (Carnation). The membrane was immunoblotted with primary antibody for 1–24 h, followed by secondary antibody coupled to horseradish peroxidase (Amersham Biosciences) for 1 h. The membrane was exposed on an x-ray film (Eastman Kodak Co.) using ECL Western blot detection reagents (Amersham Biosciences). To reprobe with different antibodies, the membrane was stripped in stripping buffer (54) at 56 °C for 1 h.

RESULTS

NFκB Activation Induced by Saturated Fatty Acid Is TLR4-dependent—As reported in our previous studies in macrophages (18, 51), saturated fatty acid (lauric acid) induced NFκB transactivation in RAW264.7 cells, and this activation was

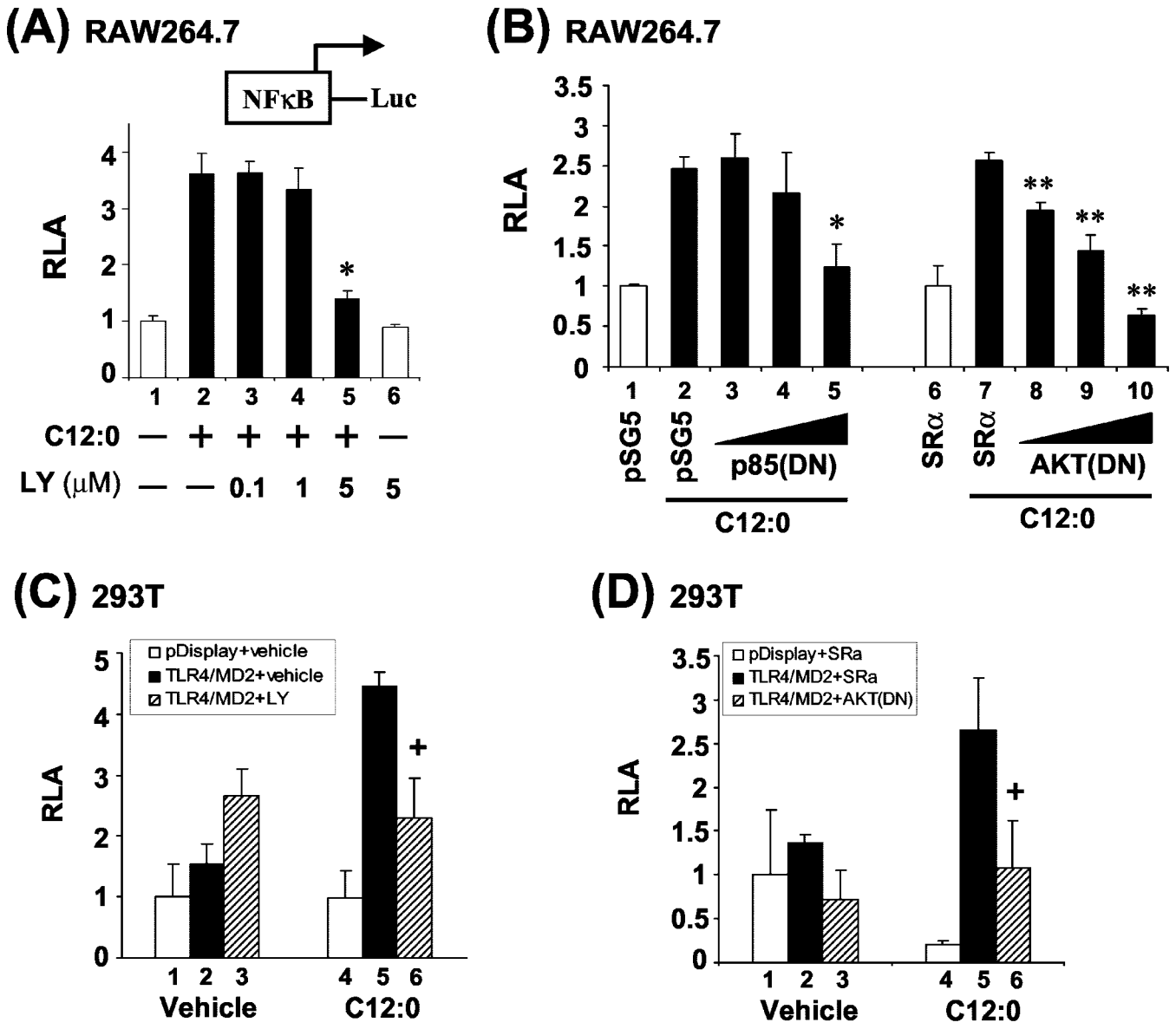

FIG. 5. **Lauric acid (C12:0)-induced NFκB activation is inhibited by LY294002 or a dominant-negative mutant of PI3K or AKT in macrophages (RAW264.7) and 293T cells transfected with TLR4 and MD2.** RAW264.7 cells transfected with the $(NF\kappa B)_2$-luciferase (*Luc*) reporter plasmid were pretreated with LY294002 (*LY*) for 30 min (*A*) or were cotransfected with the expression plasmid of a dominant-negative mutant of PI3K (p85(DN)) or AKT(DN) (1, 2, and 3 μg) (*B*) and further stimulated with lauric acid (75 μM). 293T cells transfected with the $(NF\kappa B)_2$-luciferase reporter plasmid and the expression plasmid of TLR4 and MD2 were pretreated with LY294002 (10 μM) (*C*) or were cotransfected with AKT(DN) (*D*) and further treated with lauric acid (75 μM). After 18 h, cell lysates were prepared, and luciferase activities were determined as described in the legend for Fig. 1. Data are representative of three independent experiments. Values are means ± S.E. ($n = 3$). *, +, and **, significant differences from *bars* 2, 5, and 7, respectively ($p < 0.05$). *RLA*, relative luciferase activity. SRα, vector for AKT(DN).

inhibited by a dominant-negative mutant of TLR4 (Fig. 1*A*). In this study, we further determined whether lauric acid can activate ectopically expressed TLR4 in 293T cells which do not express endogenous TLR4. The expression of TLR4 alone in 293T cells was not sufficient to induce NFκB activation by lauric acid (Fig. 1*B*). MD2 and CD14 are known to participate in the recognition of LPS by the TLR4 complex (55). Cotransfection of TLR4 and MD2 was sufficient to induce both lauric acid- and LPS-induced NFκB activation in 293T cells (Fig. 1*B*), suggesting that MD2 is required for the activation of TLR4 by saturated fatty acid and LPS. In contrast, cotransfection of CD14 with TLR4 in the absence of MD2 was not sufficient to confer lauric acid and LPS responsiveness to TLR4. However, the overexpression of CD14 with TLR4 plus MD2 resulted in a slight potentiation of NFκB activation by lauric acid or LPS as compared with TLR4 plus MD2 in 293T cells (Fig. 1*B*). A dominant-negative mutant of TLR4 inhibited lauric acid-induced NFκB activation in 293T cells transfected with TLR4 and MD2 (Fig. 1*C*). These results demonstrate that lauric acid induces the activation of endogenous or ectopically expressed TLR4 in the presence of MD2.

Saturated Fatty Acid-induced NFκB Activation Is Mediated through the MyD88/IRAK/TRAF6 Signaling Pathway—MyD88 is an immediate adaptor molecule recruited by activated TLR4, leading to the activation of signaling cascades, including IRAK-1, TRAF6, NIK, and IκB kinase (3). The activation of IκB kinase-β results in the phosphorylation of IκBα at Ser^{32} and Ser^{36} and the consequent degradation of IκBα. This degradation leads to the nuclear translocation and DNA binding of NFκB (56). We determined whether lauric acid stimulates the MyD88-dependent signaling pathways. Dominant-negative MyD88 inhibited lauric acid- or LPS-induced NFκB activation in both RAW264.7 cells (Fig. 2*A*) and 293T cells (Fig. 2*B*) transfected with TLR4 and MD2. Lauric acid-induced NFκB

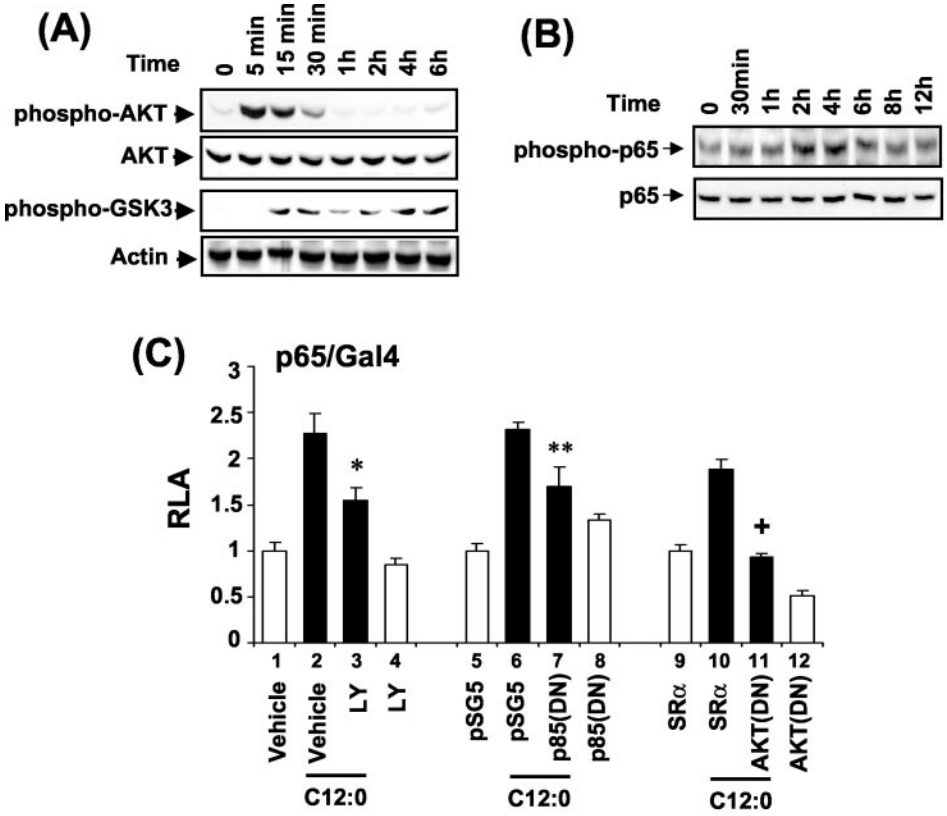

FIG. 6. **Lauric acid (C12:0) induces the phosphorylation of AKT, leading to the transactivation of p65 in macrophages.** *A* and *B*, RAW264.7 cells were treated with lauric acid (100 μM) for the indicated times, and cell lysates were analyzed by phospho-Ser^{473} AKT, AKT, phosphorylated glycogen synthase kinase-3 (*phospho-GSK3*), phospho-Ser^{529} p65, p65, or actin immunoblotting. *C*, RAW264.7 cells were transfected with the p65/Gal4 expression plasmid containing p65 fused to the DNA-binding domain of the Gal4 transcription factor and the plasmid containing the Gal4-responsive element-luciferase reporter gene. Cells were pretreated with LY294002 (*LY*; 10 μM) for 30 min or were cotransfected with the expression plasmid of a dominant-negative mutant of PI3K (p85(DN)) or AKT(DN) or a corresponding vector as indicated. Cells were further stimulated with lauric acid (75 μM) for 18 h. Cell lysates were prepared, and luciferase activities were determined as described in the legend for Fig. 1. Data are representative of two independent experiments. Values are means ± S.E. ($n = 3$). *, **, and +, significant differences from *bars 2*, *6*, and *10*, respectively ($p < 0.05$). *RLA*, relative luciferase activity. SRα, vector for AKT(DN).

activation was also inhibited by a dominant-negative IRAK-1, TRAF6, NIK, or IκBα in 293T cells transfected with TLR4 and MD2 (Fig. 2, *C* and *D*). A dominant-negative mutant of TIRAP, another adaptor molecule of TLR4 that cooperates with MyD88-dependent signaling, also inhibited lauric acid-induced NFκB activation (Fig. 2*B*). These results demonstrate that lauric acid-induced NFκB activation is mediated through MyD88-dependent signaling pathways and that MyD88, TIRAP, IRAK-1, and TRAF6 are common downstream signaling components shared by both saturated fatty acid and LPS.

The activation of TLR4 also leads to the activation of MAPKs (3). Lauric acid induced not only the degradation of IκBα, but also the transient phosphorylation of c-Jun N-terminal kinase (JNK) and extracellular signal-regulation kinase (ERK) in RAW264.7 cells (Fig. 3). These results demonstrate that lauric acid induces the activation of MAPK signaling pathways as well as NFκB activation.

Saturated Fatty Acid-induced NFκB Activation Is Also Mediated through the PI3K/AKT Pathway—LPS, a TLR4 agonist, is known to activate PI3K and AKT, a downstream target of PI3K (32). AKT is known to regulate the activation of NFκB (40–44). However, it has not been known how PI3K/AKT is involved in TLR4 signaling. We determined whether the activation of NFκB induced by TLR4 and saturated fatty acid is mediated through the PI3K/AKT signaling pathway. LY294002, a PI3K inhibitor, and a dominant-negative mutant of PI3K (p85(DN)) or AKT (AKT(DN)) suppressed NFκB activation induced by LPS in RAW264.7 cells (Fig. 4, *A* and *B*). NFκB activation induced by constitutively active (CA) TLR4 (ΔTLR4(CA)) was also inhibited by p85(DN) or AKT(DN) (Fig. 4*C*). AKT(DN) blocked MyD88-induced NFκB activation (Fig. 4*D*), suggesting that AKT is the downstream signaling component of MyD88. AKT(CA) alone was sufficient to induce NFκB activation (Fig. 4*E*). These results suggest that PI3K/AKT is the downstream signaling pathway activated by TLR4 and that AKT activation is required and sufficient for NFκB activation mediated through TLR4.

Lauric acid-induced NFκB activation was also inhibited by LY294002, p85(DN) or AKT(DN) in both RAW264.7 and 293T cells transfected with TLR4 and MD2 (Fig. 5). These results suggest that NFκB activation induced by saturated fatty acid is mediated through TLR4 and the PI3K/AKT signaling pathway. Indeed, lauric acid induced the rapid and transient phosphorylation of AKT, followed by the phosphorylation of glycogen synthase kinase-3, the substrate of AKT in RAW264.7 cells (Fig. 6*A*), demonstrating the activation of PI3K and AKT by lauric acid. The activation of AKT induces the phosphorylation and transactivation of p65, a subunit of NFκB, leading to the activation of NFκB (40–44). Thus, we determined whether lauric acid induces the phosphorylation of p65 by immunoblotting. In addition, the transactivation of p65 was determined using the p65/Gal4 plasmid containing p65 fused to the DNA-binding domain of the Gal4 transcription factor and the plasmid containing the Gal4-responsive element-luciferase reporter gene. Lauric acid induced the phosphorylation of p65 and also increased the transactivation of p65 (Fig. 6, *B* and *C*). This transactivation was inhibited by LY294002 and p85(DN) or AKT(DN) (Fig. 6*C*). These results suggest that NFκB activation induced by lauric acid is at least

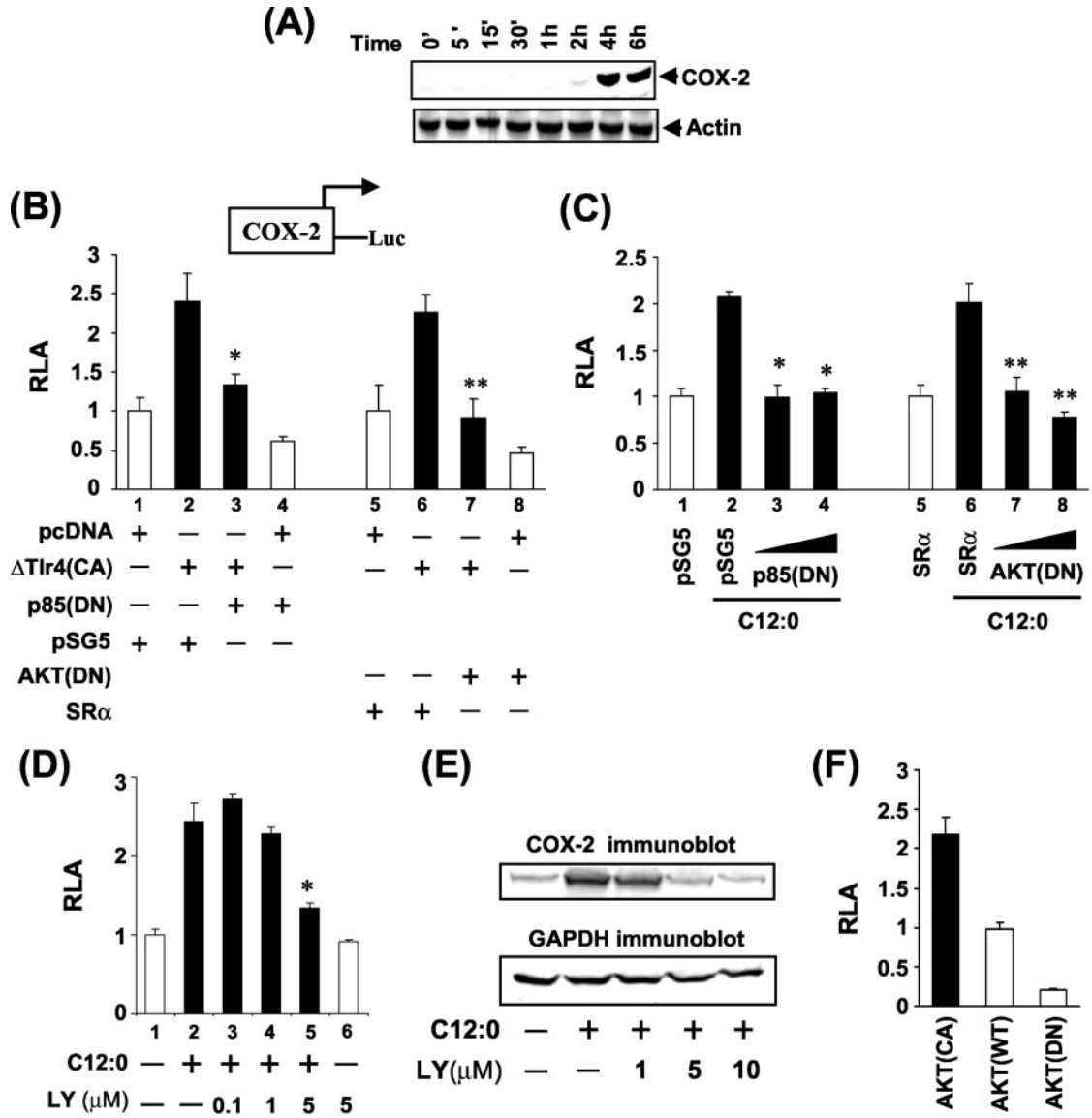

FIG. 7. **TLR4(CA)- or lauric acid (C12:0)-induced COX-2 expression is inhibited by LY294002 or a dominant-negative mutant of PI3K or AKT in macrophages.** *A*, RAW264.7 cells were treated with lauric acid (100 μM) for the indicated times, and cell lysates were analyzed by immunoblotting with anti-COX-2 antibody. *B–D* and *F*, cells were transfected with the COX-2 promoter-luciferase (*Luc*) reporter plasmid. *B*, cells were cotransfected with the expression plasmids of ΔTLR4(CA) and a dominant-negative mutant of PI3K (p85(DN)) or AKT(DN) as indicated. *C* and *D*, cells were cotransfected with the expression plasmid of p85(DN) or AKT(DN) (1, 2 μg) (*C*) or were pretreated with LY294002 (*LY*) for 30 min (*D*) and further stimulated with lauric acid (75 μM) for 18 h. *E*, cells were pretreated with LY294002 for 30 min and further stimulated with lauric acid (50 μM) for 18 h. Cell lysates were analyzed by immunoblotting with anti-COX-2 and anti-glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) antibodies. *F*, cells were transfected with AKT(CA), wild-type AKT (*AKT(WT)*), or AKT(DN). After 24 h, cell lysates were prepared. Luciferase and β-galactosidase enzyme activities were measured as described in the legend to Fig. 1. Data are representative of three independent experiments. Values are means ± S.E. ($n = 3$). * and **, significant differences from *bars 2* and *6*, respectively ($p < 0.05$). *RLA*, relative luciferase activity. SRα, vector for AKT(DN).

partly mediated through AKT activation, leading to p65 transactivation. Together, these results suggest that lauric acid activates TLR4, leading to the activation of both the MyD88/IRAK/TRAF6/NIK/NFκB and MyD88/PI3K/AKT/NFκB pathways.

COX-2 Expression by Saturated Fatty Acid Is Mediated through the TLR4/PI3K/AKT Pathway in Macrophages—COX-2 was one of the target genes that were induced by saturated fatty acid through the activation of TLR4 in RAW264.7 cells (18). The increase in COX-2 expression induced by lauric acid started at 4 h (Fig. 7*A*) and continued at least until 18 h (data not shown). We determined whether saturated fatty acid-induced COX-2 expression is mediated through the PI3K/AKT signaling pathway. p85(DN) or AKT(DN) inhibited COX-2 expression induced by ΔTLR4(CA) (Fig. 7*B*) or lauric acid (Fig. 7*C*). LY294002 also suppressed both COX-2 promoter activity and COX-2 protein expression induced by lauric acid (Fig. 7, *D* and *E*). The expression of AKT(CA) resulted in COX-2 expression (Fig. 7*F*). These results suggest that AKT activation is required and sufficient for COX-2 expression mediated through TLR4 activation and that COX-2 expression induced by saturated fatty acid is at least partly mediated through the PI3K/AKT signaling pathway.

Docosahexaenoic Acid (DHA) Inhibits the Phosphorylation of AKT and the Activation of NFκB Induced by the Activation of TLR4—The results from our previous studies showed that DHA, an *n*-3 polyunsaturated fatty acid, inhibits COX-2 expression induced by TLR4 (18). In this study, we determined whether DHA inhibits the activation of PI3K/AKT and NFκB induced by TLR4 activation. DHA suppressed the phosphorylation of AKT induced by LPS or lauric acid (Fig. 8*A*), demon-

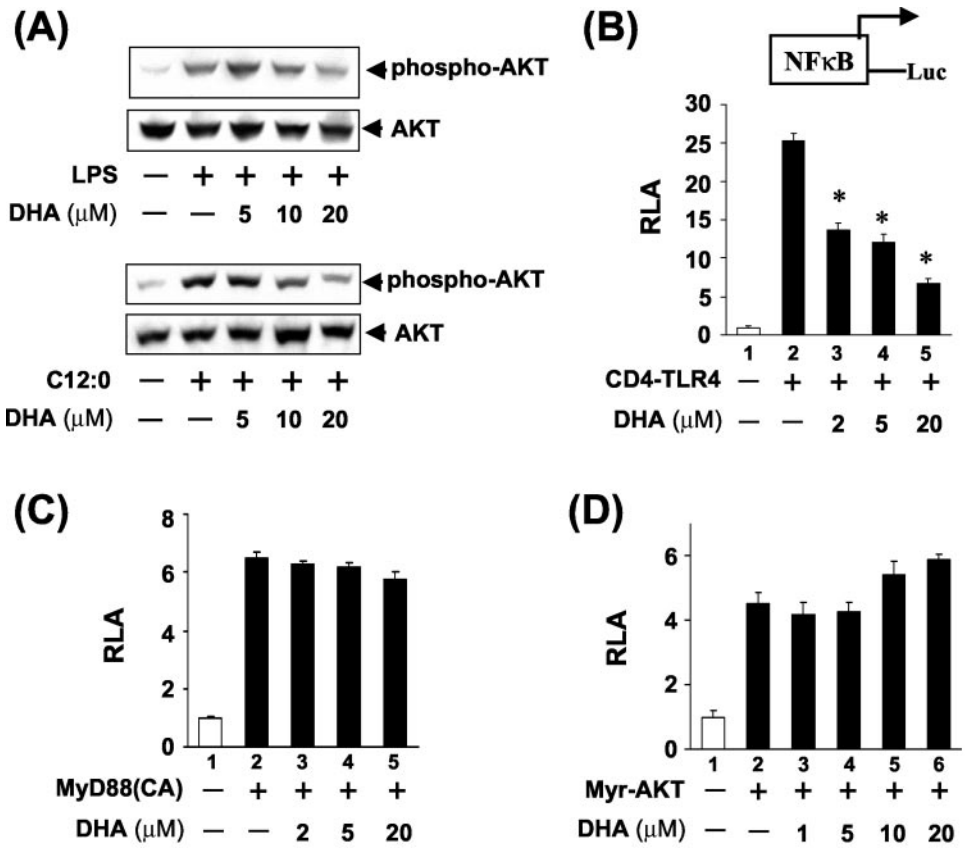

FIG. 8. **DHA inhibits the phosphorylation of AKT induced by LPS or lauric acid (C12:0) and the activation of NFκB induced by TLR4(CA), but not MyD88(CA) or AKT(CA).** *A*, RAW264.7 cells were pretreated with DHA for 2 h and further stimulated with LPS (100 ng/ml) or lauric acid (100 μM) for 15 min. Cell lysates were analyzed by immunoblotting with anti-phospho-Ser^{473} AKT and anti-AKT antibodies. *B–D*, 293T cells were transfected with the $(NF\kappa B)_2$-luciferase (*Luc*) reporter plasmid and the expression plasmid of constitutively active CD4-TLR4, MyD88(CA), or constitutively active AKT (myristoylated AKT (*Myr-AKT*)), respectively. Cells were treated with various concentrations of DHA. After 18 h, cell lysates were prepared, and luciferase activities were determined as described in the legend to Fig. 1. Data are representative of three independent experiments. Values are means ± S.E. ($n = 3$). *, significant difference from *bar 2* ($p < 0.05$). *RLA*, relative luciferase activity.

strating that DHA inhibits the activation of the PI3K/AKT pathway derived from TLR4 activation. DHA also inhibited NFκB activation induced by TLR4(CA), but not MyD88(CA) or Myr-AKT(CA) (Fig. 8, *B–D*). These results suggest that the molecular targets of unsaturated fatty acids are not the downstream signaling components of TLR4, but the TLR itself or its associated molecules.

DISCUSSION

Results from our previous studies showed that saturated fatty acids induce, but unsaturated fatty acids inhibit, the activation of TLR4 as determined by NFκB activation and COX-2 expression (18, 51). These results suggest the novel role of dietary fatty acids as a modulator of TLR4 signaling. However, the downstream signaling pathways through which the fatty acids modulate NFκB activation and target gene expression have not been determined. Different TLR agonists may induce the activation of different downstream signaling pathways, leading to the diverse array of target gene expression and cellular responses. Therefore, in this study, we determined how the TLR4 signaling pathways are regulated by saturated and unsaturated fatty acids. Tentative downstream signaling pathways derived from TLR4 activation that are modulated by fatty acids are illustrated in Fig. 9. The results in this study demonstrate that saturated fatty acid and LPS share the common signaling pathways of TLR4 involving the MyD88, IRAK-1, TRAF6, and PI3K/AKT pathways, leading to NFκB activation and target gene expression, including COX-2. These results reinforce the possibility that both saturated fatty acids and LPS interact with the same receptor complex. Furthermore, these results provide an important clue to elucidate the molecular mechanisms by which LPS and saturated fatty acids induce target gene expression and the consequent cellular responses through the TLR4 complex. Our results also establish that saturated and polyunsaturated fatty acids reciprocally modulate the TLR4 signaling pathways; saturated fatty acid induces phosphorylation of AKT and activation of NFκB through TLR4 activation, whereas unsaturated fatty acid inhibits TLR4-induced AKT phosphorylation and NFκB activation.

The activation of NFκB is known to be regulated by at least two different mechanisms: the phosphorylation and consequent degradation of the inhibitor protein IκBα and the phosphorylation of p65, a subunit of NFκB, in a cooperative manner (40, 56–58). IκBα is phosphorylated by IκB kinase activated through the MyD88/IRAK/TRAF6 pathway and subsequently degraded, resulting in nuclear translocation and DNA binding of NFκB. p65 is phosphorylated by serine kinases, including protein kinase A (Ser^{276}) and IκB kinase (Ser^{536}) (42, 43). The phosphorylation of p65 is critical for the interaction with the coactivator proteins p300 and CBP to promote the transcriptional activity of NFκB (43, 59). PI3K/AKT signaling has been suggested as one of the regulators for NFκB activation induced by inflammatory stimuli such as tumor necrosis factor, interleukin-1, and LPS (32, 40, 60, 61). Several studies demonstrated that AKT can enhance the transcriptional activity of NFκB through the phosphorylation of p65 independent of IκBα degradation (40–44). Our results demonstrated that, in addition to the degradation of IκBα (Fig. 3), lauric acid induces the

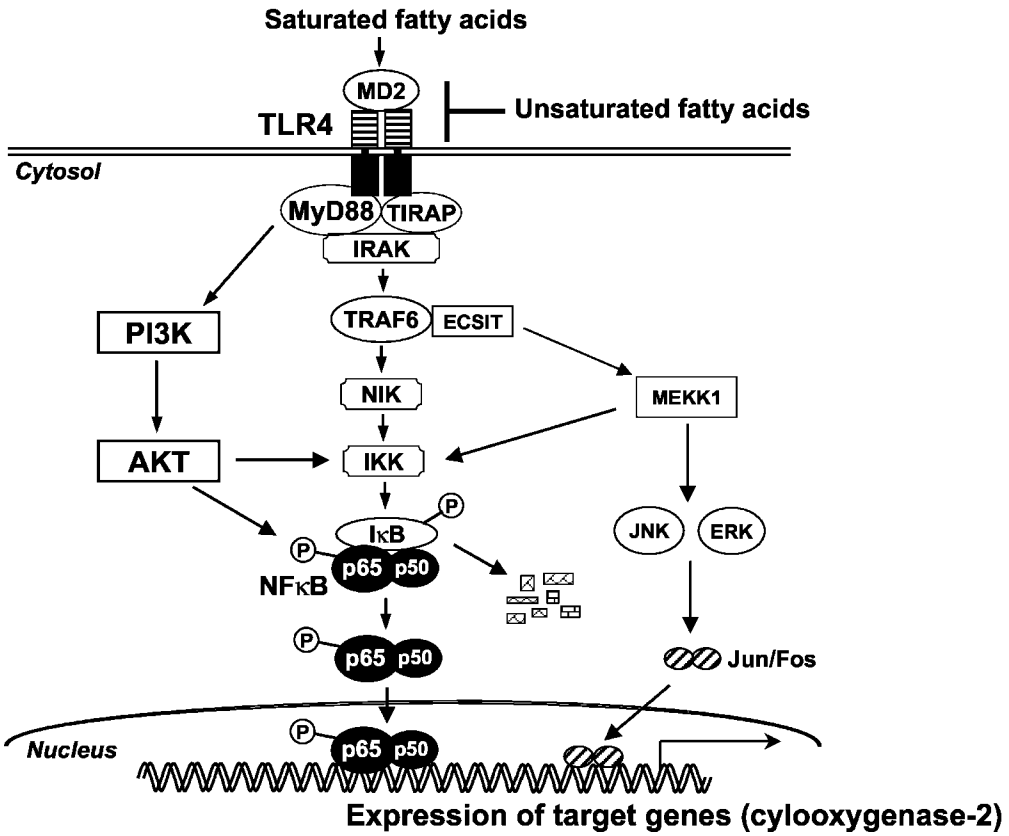

FIG. 9. **TLR4-mediated downstream signaling pathways leading to the expression of target genes, including COX-2, and reciprocal modulation by saturated and unsaturated fatty acids.** Our results suggest that saturated fatty acids activate PI3K/AKT as well as the common MyD88-dependent signaling pathway, including IRAK/TRAF6/NIK, leading to the activation of NFκB and MAPKs and the expression of target genes, including COX-2. In contrast, unsaturated fatty acids inhibit the phosphorylation of AKT and the activation of NFκB induced by TLR4 activation. *ECSIT*, evolutionarily conserved signaling intermediate in Toll pathways; *MEKK1*, MAPK/ERK kinase kinase-1; *IKK*, IκB kinase.

serine phosphorylation of p65 at Ser^{529} and the transactivation of p65 (Fig. 6). p65 transactivation was associated with AKT activation induced by saturated fatty acid (Fig. 6). This is consistent with a previous report that the activation of p65 by AKT is accompanied by the phosphorylation of p65 at Ser^{529} (43). Because the magnitude of IκBα degradation by lauric acid was mild, it is possible that the phosphorylation of p65 may be a major mechanism by which saturated fatty acid promotes NFκB activation. Together, these results suggest that the full activation of NFκB by saturated fatty acids requires both IκBα degradation via the MyD88/IRAK/TRAF6 pathway and p65 transactivation by the PI3K/AKT pathway.

Synthetic bacterial lipopeptides (TLR2 agonist) and CpG DNA (TLR9 agonist) can neither activate NFκB nor induce the expression of inflammatory cytokines in macrophages derived from MyD88-deficient mice (62–64). However, unlike TLR2 and TLR9 agonists, LPS can still induce the activation of NFκB and MAPKs, the production of interleukin-18, and the maturation of dendritic cells in a delayed fashion in MyD88-deficient cells (64–67). These results suggest that TLR4 is coupled to an additional signaling pathway that is independent of MyD88. TIRAP/Mal has been identified as an adaptor molecule associated with TLR4, leading to the activation of IRAK-1 and NFκB (26, 27). It has been suggested that TIRAP may mediate the MyD88-independent signaling pathway. However, it was recently demonstrated that LPS-induced expression of interferon-inducible genes such as IP-10, GARG-16, and IRG-1 was still observed in macrophages derived from MyD88 and TIRAP double-knockout mice (28). These results suggest that TIRAP cooperates with MyD88 and participates in the MyD88-dependent signaling pathway and that there may be additional adaptors responsible for the MyD88-independent signaling pathway. Recently, TRIF/TICAM was reported to mediate the MyD88-independent signaling pathway of TLR3 (30, 31). Our results show that both MyD88 and TIRAP are involved in the signaling pathways activated by saturated fatty acid. It remains to be determined whether saturated fatty acid can also activate MyD88-independent signaling components like TRIF.

The PI3K/AKT pathway was suggested as one of the downstream signaling pathways of TLRs. Wortmannin, a PI3K inhibitor, inhibits NFκB activation and cytokine production by CpG DNA, a TLR9 agonist (68). The activation of NFκB and AP-1 induced by LPS, a TLR4 agonist, is also inhibited by wortmannin (69). B lymphocytes deficient in PI3K regulatory units (p85α, p55α, and p50α) fail to respond to LPS (70). Arbibe *et al.* (34) demonstrated that TLR2 stimulation by *Staphylococcus aureus* induces the recruitment of PI3K and the activation of AKT, leading to NFκB transactivation. These results demonstrate the role of PI3K in TLR signaling pathways. However, unlike TLR2, TLR4 does not have a PI3K-binding site. Therefore, it has not been clear how TLR4 activation leads to PI3K/AKT activation and whether TLR4-mediated AKT activation is MyD88-dependent or -independent. Our results show that AKT(DN) inhibited NFκB activation induced by MyD88(CA) (Fig. 4*D*). MyD88 has a binding motif for p85, a regulatory unit of PI3K. Recently, Ojaniemi *et al.* (71) reported that PI3K can be associated with MyD88 in response to LPS in mouse macrophages. Together, these results suggest that PI3K/AKT is the downstream signaling component of the MyD88-dependent TLR4 signaling pathway.

The recognition of LPS is mainly mediated through the interaction with three proteins, TLR4, CD14, and MD2. LPS

binds to the serum protein LPS-binding protein, and the LPS·LPS-binding protein complex can be recognized by CD14. CD14, a cell-surface 55-kDa glycoprotein with a glycosylphosphatidylinositol membrane anchor, is present as a soluble form in blood or as a membrane-bound form in myeloid lineage cells (72, 73). Because CD14 does not have a cytosolic domain, it has been suggested that CD14 interacts with other receptors to transduce the LPS signal into cells. Recently, TLR4 was identified as an LPS receptor (10, 74, 75), and it has been further shown that LPS can induce physical proximity between CD14 and TLR4 using fluorescence resonance energy transfer techniques (76). MD2 is a 20–30-kDa glycoprotein that binds to the extracellular domain of TLR4 (55). TLR4 and MD2 provide greater specificity for ligands and more efficient responsiveness to LPS (55). A recent study also showed that LPS is in close proximity to TLR4 in the presence of CD14 and MD2 (77). Our results show that cotransfection of TLR4 with MD2 was sufficient to induce NFκB activation induced not only by LPS, but also by lauric acid in 293T cells, suggesting that MD2 is required for both saturated fatty acid- and LPS-induced TLR4 signaling. Although cotransfection of CD14 with TLR4 in the absence of MD2 did not confer the responsiveness of LPS or lauric acid to TLR4, the cotransfection of CD14 with TLR4 plus MD2 resulted in a slight potentiation of NFκB activation by lauric acid or LPS compared with TLR4 plus MD2 in 293T cells. The serum in the culture medium contains soluble CD14, and both soluble CD14 and membrane-bound CD14 can function to confer LPS responsiveness (72, 78). Therefore, our experimental conditions do not allow us to conclude whether CD14 is required for saturated fatty acid-induced TLR4 activation. We cannot exclude the possibility that CD14 also contributes to the activation of TLR4 by saturated fatty acid. Together, our results demonstrate that the responsiveness of lauric acid to the combination of TLR4, MD2, and CD14 is similar to that of LPS, suggesting that both saturated fatty acid and LPS activate the signaling pathways through the same receptor complex. The results that unsaturated fatty acids inhibit NFκB activation induced by TLR4, but not the downstream signaling components, suggest that the inhibitory target of unsaturated fatty acids is TLR4 or its associated molecules. However, the detailed molecular mechanism by which the fatty acids modulate TLR activation is not known. The molecular mechanism by which LPS interacts with the TLR4 complex is also not well understood, although it has been shown that LPS stimulation leads to close proximity between TLR4, CD14, and MD2 (77). Whether saturated fatty acids induce and unsaturated fatty acids inhibit the physical proximity between TLR4, CD14, and MD2 to modulate TLR signaling needs to be determined in the future studies.

COX-2 is overexpressed in sites of inflammation and in many types of tumors and is involved in tumorigenesis and angiogenesis (50, 79–82). Our results demonstrating that saturated fatty acid induces, but DHA inhibits, NFκB activation and COX-2 expression suggest the possibility that the risk of tumors and inflammatory diseases can be differentially modulated by types of dietary fatty acids. Together, these results suggest the possibility that TLR-mediated target gene expression and cellular responses may be differentially modulated by saturated and unsaturated fatty acids through the reciprocal modulation of TLR signaling pathways.

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